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Mediator Release after Nasal Airway Challenge with Allergen[1-4]

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SUMMARY An *in vivo* model of human allergic disease has been developed in which nasal challenge with antigen leads to physiologic changes, together with a release of increased amounts of inflammatory mediators into nasal secretions obtained by washing the nose with saline. In 105 experiments involving 35 subjects, only allergic subjects consistently demonstrated an increase in the concentrations of the mast cell mediator, histamine, and the putative mast cell mediators, TAME-esterase and PGD_2. The release of each mediator was significantly ($p < 0.001$) related to the physiologic change (sneezing). The release of each mediator also correlated significantly with the release of the other 2 mediators ($p < 0.001$). This system, for the first time, clearly relates an *in vivo* symptom and mediator release and thus should provide an excellent tool for the further study of the allergic response and nasal pathophysiology.

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Introduction

The pathogenesis of allergic rhinitis is presumed to involve an IgE-mediated mechanism. The exposure of a patient to an antigen leads to the formation of IgE antibodies, which in turn bind to receptors on mast cells and basophils. Subsequent exposure to antigen causes cross-linking of specific IgE antibodies on these cells and the release of inflammatory mediators. The mediators interact with neural elements, mucosal glands, and blood vessels to cause sneezing, rhinorrhea, and nasal congestion, which represent the major physiologic responses associated with allergic rhinitis (1).

Deliberate challenges go back to 1873 when Blackley placed grass pollen in the nose of susceptible subjects and induced the signs and symptoms of allergic rhinitis (2). Subsequent studies of nasal responses after antigen challenge have included attempts to quantitate the reaction by measurements of airway resistance (3-5), mucus production (6), and subjective assessment (7). Unfortunately, these parameters are subject to marked physiologic variability before, during, and after challenge (8, 9), and there are also technical difficulties associated with each measurement (10, 11).

Studies to assess the role of inflammatory mediators in allergic rhinitis have been straightforward: investigators tried either to recover them from nasal secretions after antigen challenge or placed chemically defined mediators into the nose to see if they duplicated the response to antigen. In 1970, Dolovich and coworkers (12) demonstrated kininlike activity in the nasal secretions of 7 of 12 allergic subjects after antigen challenge. In 1977, Okazaki and associates (13) collected nasal secretions from symptomatic hay fever patients and found prostaglandin E (PGE) in 6 of 12 subjects. And in 1978, Eggleston [illegible] found histamine in nasal washes but observed no difference between asymptomatic allergic and nonallergic subjects. The only inflammatory mediators that have been applied to the nose are histamine (15) and PGE (16). Although these substances can cause some physiologic alterations, they have not reproduced all of the physiologic changes of allergic rhinitis. Because multiple mediators are released from mast cells and basophils, this is not unexpected.

We report here the first experiments that correlate the clinical response with the release of mediators during and after antigenic challenge of the nose. We demonstrate that the increased concentrations of histamine, TAME-esterase, and prostaglandin D_2 (PGD_2) found in nasal wash fluids after nasal provocation with antigen correlate significantly with the appearance of a symptom, sneezing. This is perhaps the most definitive demonstration that antigen administration *in vivo* leads to the release of mast cell mediators.

Methods

Subjects

We studied healthy male and female volunteers between 18 and 40 yr of age who gave informed consent prior to study. Allergic subjects were defined as having seasonal symptoms to an antigen to which they had a positive intradermal skin reaction at a concentration of 10 PNU/ml or less of crude allergen extracts. Nonallergic subjects had no seasonal symptoms and had negative skin tests to the antigen extracts (100 PNU/ml).

Reagents

Ragweed pollen, ragweed extract, and mixed grass extract were purchased from Greer Laboratories, Lenoir, NC. Normal saline (Cutter Laboratories, Emeryville, CA) and oxymetazoline hydrochloride (Afrin®, Schering Corp., Kenilworth, NJ) were purchased.

Collection of Nasal Secretions

The subjects extended their necks approximately 30° from the horizontal while in a sitting position. Five milliliters of normal saline (0.9%) were instilled into each nostril while the subjects did not breathe or swallow. After 10 s, the subjects flexed their necks, expelling the mixture of mucus and saline into a vessel, which was stored on ice

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until the conclusion of the experiment. The wash fluid was centrifuged at 4° C for 15 min at 15,000 × *g*, after which the sol phase was separated from the gel phase by pipetting. Samples were stored at −20° C until assayed.

Mediator Assays

Histamine. For histamine determinations, 0.8 ml of sample was combined with 0.2 ml of 8% $HClO_4$ and centrifuged for 10 min at 1,000 × *g*. The supernatants were assayed by an automated spectrofluorometric assay (17). Concentrations of histamine of ≥ 1 ng/ml can be measured with an accuracy of ± 5%. In order to confirm that histamine was being measured in nasal washes, 3 types of experiments were performed. First, samples with measurable concentrations of histamine were found to dilute concordantly with known histamine standards. Second, known histamine standards added linearly to the samples. Third, diamine oxidase destroyed measurable histamine in nasal washes.

TAME-esterase. Enzyme(s) having arginine esterase activity were measured by a modification of the radiochemical assay developed by Beaven and colleagues (18), which is based on the liberation of H^3-labeled methanol from the synthetic substrate, H^3 TAME: 40-μl aliquots of sample were added to a polypropylene microtube containing 10 μl of a 0.2 M Tris buffer (pH, 8.0) and the reaction was initiated by the addition of 10 μl of H^3 TAME (0.035 μCi). The microtube was then immediately suspended in 10 ml of Econofluor (New England Nuclear, Boston, MA) and 50 μl of stop solution (1 vol glacial acetic acid to 10 vol 0.02 M TAME) in a closed counting vial. The microtube was incubated for 1 h at room temperature in the closed vial before the reaction was terminated by shaking. The vial was then counted in a liquid scintillation spectrometer for 4 min. Enzymes having activity in this assay are designated as TAME-esterases.

Prostaglandin D_2. The PGD_2 was measured by a competitive radioimmunoassay based on previously described assay systems (19). The anti-PGD_2 was kindly provided by Dr. Levine, Brandeis University. The cross-reactivity of the antiserum was less than 1% with PGE_1, PGE_2, TxB_2, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$. The PGD_2 standards added linearly to nasal washes, and nasal washes with PGD_2 diluted concordantly with standards.

Extractions

The distribution of PGD_2 and histamine between the sol and the gel phase of the nasal washes was studied. The gel phase could not be directly assayed because of the inability to obtain adequate aliquots; hence, both phases were extracted as follows.

Histamine. Two and a half ml of *n*-butanol and 300 ng of NaCl were added to 1.0 ml of sample, 0.2 ml of 3 N NaOH was then added, and the mixture was shaken for 1 min. The sample was then centrifuged at 1,000 × *g* for 5 min; 1.5 ml of the top (butanol) layer were removed and combined with 1.2 ml of 0.12 N HCl plus 1.9 ml *n*-heptane. The mixture was then vortexed for 1 min. A sample from the bottom layer (acid) was assayed for histamine as described above.

PGD_2. Four ml of 95% ETOH were combined with 1.0 ml of the sample and vortexed for 1 min. After centrifugation at 1,000 × *g*, the supernatant was vacuum-extracted to dryness. The sample was reconstituted to 1 ml with 0.1% gelatin-PBS and assayed as described above.

The mediators measured were found to be essentially equally distributed between the sol and the gel phase, so for convenience all measurements were performed on the sol phase, except where indicated.

Antigen Delivery System and Nasal Airway Resistance

Antigen delivery. Pollens were insufflated in the nose by a technique similar to that described by Rosenberg and coworkers (20). In brief, varying amounts of ragweed pollen were combined with lactose to obtain a total weight of 28.4 mg and placed in a gelatin capsule. The capsule was placed in a spinhaler® (Fisons, Bedford, MA) that was adapted for nasal application and connected to a French-Rosenthal dosimeter. When the dosimeter was activated (4 times for 2.0 s with a driving pressure of 10 psi), about 25 mg of pollen and lactose (± 15%) were expelled from the spinhaler into the nose. When antigen extracts were used, they were placed in a DeVilbiss 646 nebulizer (DeVilbiss, Somerset, PA) that was adapted to fit into a nostril. Like the spinhaler it was attached to a dosimeter that, when activated, carried the extract into the nose delivering 0.08 ml of extract from the nebulizer.

Nasal airway resistance. Nasal airway resistance (NAR) was measured in both nostrils by anterior rhinomanometry. A flow probe was inserted into one nostril and a pressure probe inserted into the other. The probes were reversed to measure NAR in the second nostril. Each breath generated a tracing on an X-Y recorder; NAR was calculated during expiration by dividing pressure (measured in cm H_2O) by flow (measured in liters per second). For flows greater than or equal to 20 L/s, the pressure was measured at a flow of 20 L/s. When the flow was less than 20 L/s, the pressure was fixed at 20 mm H_2O and the flow was determined. Conductance (NAR^{-1}) is the reciprocal of NAR.

Patient evaluation. The subjects were able to perform their routine activities upon leaving the laboratory. Frequently, minor local symptoms persisted for hours. Three patients experienced mild epistaxis, which stopped without treatment. Rarely, an aerosol of antigen extract was inhaled into the lower respiratory tract causing mild wheezing. This did not occur with whole pollen challenge. No episodes of acute otitis media or sinusitis have occurred.

Results

In preliminary experiments, we were consistently able to elicit an allergic response by nasal antigen challenge, but 3 problems became apparent: first, the prechallenge washes contained measurable amounts of histamine and TAME-esterase; second, washing the nose with saline had an unpredictable effect on NAR; third, whereas in 100 consecutive nasal washes prior to antigen challenge the instillation of 5 ml of normal saline into each nostril led to a recovery of 8.1 ± 1.1 ml of nasal wash fluid, the mucosal edema induced by the allergic response frequently reduced the capacity of the nose to accept instillation of 10 ml of saline and thereby reduced the recovery after antigenic challenge in a variable fashion.

With respect to the first problem, we established that the mediators found in the first nasal washes were not cell associated. The values were the same before and after centrifugation, filtration, and cell lysis by $HClO_4$. The problem was solved, for the purposes of these experiments, by the finding that several nasal washes reduced the concentration of mediators to consistently low or undetectable values so that challenge could be initiated.

We were not able to solve the second problem. Consequently, NAR was measured but was not considered a quantitative parameter; NAR data are, however, presented for illustrative purposes. The clinical response was assessed by counting the number of sneezes after antigen challenge.

As will be described below, we dealt with the third problem by pretreatment with a vasoconstrictor drug to reduce mucosal edema. In figure 1A, a unilateral nasal challenge with ragweed in an allergic subject without premedication is presented. Several prechallenge nasal washes reduced the concentration of histamine to baseline; in this experiment, the TAME-esterase level was not elevated in the initial wash. The variable effect of nasal washes on NAR can be seen. A placebo challenge with lactose (in some experiments, diluent) was administered after the initial nasal washes. This maneuver was performed

to evaluate the effect of the delivery system on the release of mediators into the nasal washes. In 105 experiments, sneezing occurred 8 (7.6%) times during placebo challenges. In 2 of these 8 experiments where sneezing occurred, mediator concentrations increased threefold from the prior wash. Thus, the method of delivery affected the concentration of mediators in less than 2% of the experiments performed (2 of 105).

Ragweed pollen (in figure 1A) induced sneezing and total nasal obstruction. The nasal wash 10 min after antigen stimulation had an increased concentration of TAME-esterase. Although the histamine concentration appears to increase, this must be interpreted cautiously because the precision of the histamine assay is about 1 ng/ml. In 2 additional subjects, nasal washes could be performed after antigen challenge and before nasal obstruction; increases in mediators could be demonstrated. Because the nasal congestion usually precluded adequate washes, the third problem was addressed by using a pharmacologic agonist (an α-adrenergic agent) to block the vascular response.

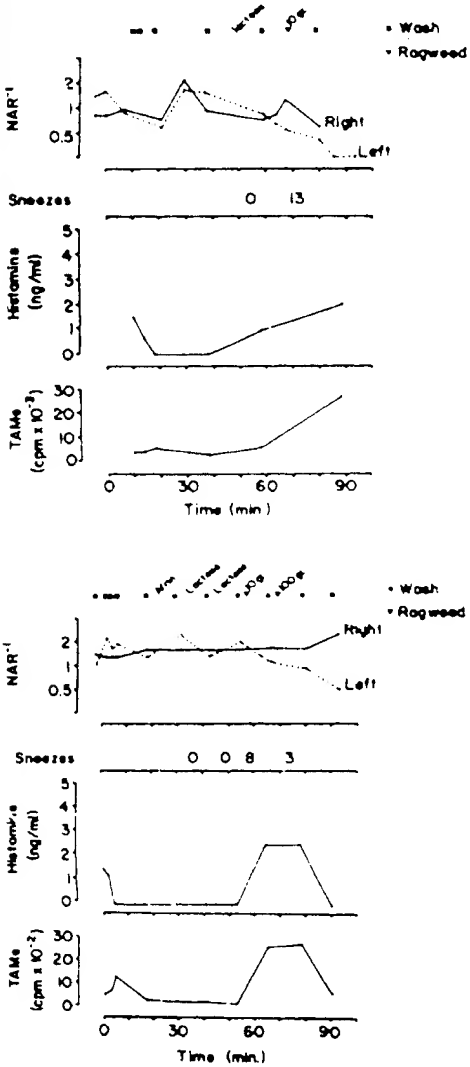

Fig. 1. Allergic subject challenged with ragweed pollen – left nostril. A. Changes in nasal airway resistance, sneezing, and mediator levels over time during nasal challenge. The experimental protocol is shown at the top of the figure. (NAR⁻¹ = conductance. ■ = saline lavage. ▼ = pollen challenge)

An experiment performed on the same subject shown in figure 1A is represented in figure 1B. The difference is that after the initial washes, 0.08 ml of oxymetazoline hydrochloride 0.05% (Afrin®) was applied topically to the nasal mucosa. The drug did not alter the baseline concentrations of either histamine or TAME-esterase in any of 105 experiments. In addition, the response to 10 grains of ragweed pollen induced sneezing that was associated with an increased concentration of histamine and TAME-esterase of the same order of magnitude as occurred in the first experiment. Multiple experiments of this type were not performed because of the difficulty in finding subjects whose nasal volume was not significantly decreased by the allergic response. However, in 2 additional patients with nasal volumes large enough to allow postchallenge washes, mediator levels with and without Afrin were similar.

Other advantages accrued to the use of the adrenergic agent as shown in figure 1B. First, because nasal volume was not decreased after the first challenge, it allowed a second dose of pollen to be delivered, which again induced sneezing, and mediators remained measurable. Note, however, that despite premedication with an α-adrenergic agonist, NAR declined after the 100 pollen grain challenge. Second, a nasal wash performed 22 min postchallenge demonstrated a return in mediator concentrations toward baseline. Oxymetazoline induced one or two sneezes in 15% of the experiments. However, in no experiment employing oxymetazoline did the concentration of mediators increase significantly over the value in

The time course of mediator disappearance after a single dose of antigen in 3 subjects is shown in figure 2. The concentrations of mediators are expressed as the percentage of the highest value obtained. Washes were begun 6 min after antigen application because this was the interval necessary for sneezing to subside. In these and subsequent experiments, we measured the major mast cell cyclooxygenase product, PGD_2, as well as histamine and TAME-esterase. The PGD_2 and histamine levels returned to baseline within 24 min, whereas the concentration of TAME-esterase was still elevated at this time.

In order to demonstrate that the responses observed in allergic subjects were specific for antigen and not caused by some nonspecific property of the antigen, nonallergic subjects were challenged. Eight allergic subjects and 8 age- and sex-matched nonallergic subjects were challenged using similar protocols. Two additional allergic subjects served as their own controls because they were challenged with an antigen to which they were not allergic. The protocols varied with respect to the antigen administered (mixed grass extract, ragweed extract, and ragweed pollen) and the presence or absence of oxymetazoline hydrochloride pretreat

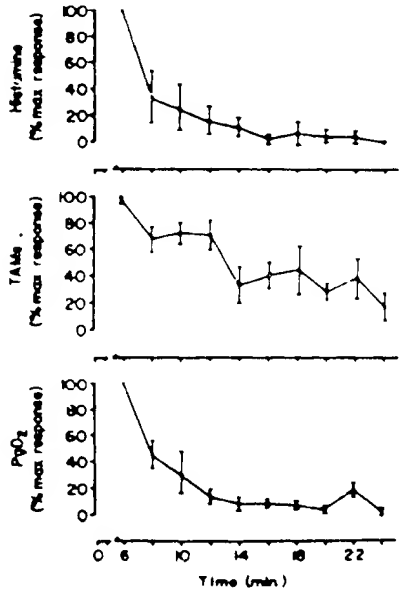

Fig. 2. The time course of the disappearance of 3 mediators after sequential nasal lavages. A single dose of antigen was applied 6 min prior to the first

ment. By Wilcoxon's paired analysis, sneezing and the concentrations of histamine, TAME-esterase, and PGD_2 in nasal washes were significantly elevated in allergic subjects challenged with relevant allergenic extracts when compared with nonallergic subjects ($p < 0.001$).

The data obtained on these 10 patient pairs are shown in figure 3. The number of sneezes and the maximal *change* in the postchallenge concentration of the 3 mediators is depicted.

The results for sneezing are apparent. None of the normal control subjects sneezed, whereas 3 to 17 sneezes occurred after antigen challenge in the allergic subjects. Although the number of responders and the mean levels of histamine released into nasal washes were greater in the allergic group, considerable overlap existed. This was due, in part, to the noise-to-signal ratio in the assay system itself and also to the occasional subject with a high baseline level of histamine despite multiple washes. Of the mediators measured, the TAME-esterase results showed the clearest difference between allergic and normal control subjects, with only 1 allergic subject showing levels in the "normal range." Again, considerable overlap occurred with regard to PGD_2, although group differences were clear. This is the most sensitive of the mediator assays, with 0.3 pg/0.1 ml being clearly positive. Thus, very small increases in PGD_2 in the nasal washes from normal subjects can be detected. Also, although we tried to eliminate drug use for 48 h prior to the challenges, the low values obtained in some allergic subjects might have been due to consumption of cyclooxygenase inhibitors, such as aspirin.

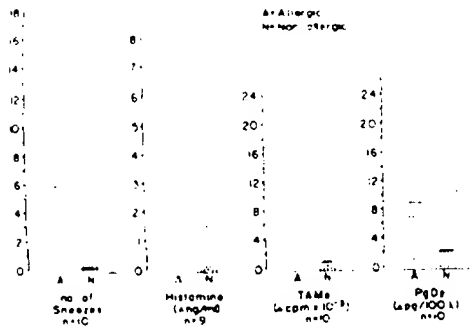

Fig 3. A comparison of sneezing and mediator release in 10 matched allergic (A) and nonallergic (N) subjects. Because of inadequate sample size, only 9 values are shown for histamine. The dots indicate the maximal number of sneezes or the maximal change in concentration of each mediator in the nasal washes. The arithmetic means are shown by the lines.

Because of the variation in baseline values and in assay sensitivities, it was decided, prospectively, on the basis of our preliminary data, to make the following definitions of a positive response. Sneezing was defined as positive when it occurred 3 or more times after antigen administration. Mediator release was defined as positive: (*1*) if a threefold increase occurred in a nasal wash after antigen challenge when compared with the levels in the fluids after the last placebo challenge, or (*2*) if the baseline was negative, any increase in mediator concentration occurred after challenge. Using these criteria, we analyzed the data from 13 experiments on nonallergic subjects; no sneezes occurred nor did positive changes in mediator concentrations occur. In 72 of 81 experiments performed on 21 allergic subjects, a positive clinical response was associated with an increase in the concentration of one or more of the mediators studied. Four subjects with a positive skin test but no seasonal symptoms were also evaluated. Two repeatedly sneezed after antigen challenge and also released mediators. The other 2 had neither a clinical response nor increases in mediators in nasal wash fluids.

In these and subsequent experiments, we found that the physiologic response of sneezing was highly correlated with the recovery of histamine, TAME-esterase activity, and PGD_2 in nasal wash fluids (table 1). The data analysis was based on 2 specimens chosen as described above from each of the 105 experiments. Not all mediators were measured in each pair of samples. When sneezing occurred, 58% of the patients released histamine, 86% released TAME-esterase, and 71% released PGD_2. The appearance of mediators in nasal washes when sneezing did not occur was infrequent, ranging from 2 to 5% of the samples for all 3 mediators. When sneezing and the release of the 3 mediators were compared, all correlations were highly significant by Fischer's exact test ($p < 0.001$).

As shown in table 2, the appearance of each mediator (histamine, TAME-esterase, and PGD_2) in wash fluids was also highly correlated with detection of each other mediator ($p < 0.001$). The TAME–esterase activity was most consistently found when another mediator appeared and was, itself, the mediator most often measurable when PGD_2 or histamine were not present. The converse of this positive concordance of mediator release was also true. In other words, when one mediator was not present, the others were also unmeasurable in the wash fluids. For example, when histamine was not released, neither were TAME-esterase or PGD_2 in 60 and 64% of all experiments (data not shown).

Discussion

In our attempts to understand and treat human allergic disorders, it is necessary that we have systems in which we can assess the *in vivo* relationship between mast cell mediator release and antigenic stimulation. To date, attempts to measure mediator release *in vivo* have largely involved taking blood specimens after antigen bronchoprovocation. This has led to confusing results, with some groups reporting the release of mediators and others failing to find them (21–23). Plasma histamine levels have most frequently been measured, but the results are complicated by the fact that a recent study demonstrates that many laboratories carrying out these measurements are unable to do so accurately (24). Indeed, it is known that blood levels of histamine over a few nanograms per milliliter lead to hypotension, but some investigators have reported values of 10 to 100 ng/ml without systemic clinical symptoms. The measurements of neutrophil chemotactic factors after challenge seem somewhat more congruous, but this work is in its early stages and it is not clear that a neutrophil chemotactic factor is a mast cell mediator (25).

The nose is readily available, and specimens may be obtained by noninvasive techniques, but there have been few attempts to study mediator release

TABLE 1

CORRELATION BETWEEN ALLERGEN-INDUCED SNEEZING AND MEDIATORS IN NASAL WASH FLUID

Physiologic Response (Sneezing)	Mediators		
	Histamine	TAME-Esterase	Prostaglandin D_2
Positive	46/79* (58%)†‡	67/78 (86%)‡	29/41 (71%)‡
Negative	4/129 (3.1%)	6/132 (4.5%)	1/65 (1.5%)

* Positive/negative
† % positive
‡ $p < 0.001$ compared with negative physiologic response

TABLE 2

CONCORDANCE OF MEDIATOR RELEASE AFTER NASAL CHALLENGE

Mediator Present	Coincidental Positive Tests (%)		
	Histamine	TAME Esterase	PGD_2
Histamine	-	100% (48/48)*	84% (21/25)
TAME	58% (48/83)	-	78% (38/49)
PGD_2	60% (21/35)	97% (38/39)	-

* Number positive/total tests

in the nose and these have not, by and large, been definitive. The studies of Dolovich and coworkers (12) with kinins are incomplete in that they were unable to obtain adequate samples from nonallergic subjects or from allergic subjects prior to challenge. Also when activity was found after challenge, it occurred in only half of the allergic subjects. Finally, it was not clear what the kininlike activity was; i.e., whether it represented plasma leakage of kallikrein acting on plasma kininogen or involved the generation of mast cell products. Okazaki and associates (13) measured PGE in secretions from allergic patients during the pollen season. This prostaglandin was found in pooled nasal washes from control subjects and in only half of the allergic subjects. It is now known that human mast cells do not generate PGE (26). Finally, Eggleston and coworkers (14), like ourselves, found histamine in the nasal washes of all subjects, whether asymptomatic allergic or nonallergic, prior to antigen challenge.

The present results show a clear-cut relationship between the appearance of increased amounts of 3 mediators, most likely derived from mast cells, and the clinical symptom of sneezing observed after antigenic nasal challenge. Whether this antigenic challenge adequately reflects field exposure is moot. It should be noted, however, that many patients responded when only 10 grains of ragweed pollen were blown into the nose. Our data demonstrated that the appearance of histamine, TAME-esterase, and PGD_2 in nasal washes was highly correlated with the clinical symptom of sneezing. Of these mediators, only histamine is strictly limited to basophils and mast cells. However, PGD_2 seems to be pregenase product released. In studies with antigen challenge of human lung tissue, for example, essentially all of the PGD_2 released was shown to originate from the mast cells (27). The TAME-esterases also come from mast cells and basophils, but there are many TAME-esterases in other cell types and in serum (28). Our preliminary work indicates that the TAME-esterase found in nasal secretions has the characteristics of the mast cell enzyme and is dissimilar from glandular and serum esterases (29). The fact that there is a high correlation between the appearance of histamine and the other 2 mediators suggests, but does not prove, that they all originate from the same cell source.

The present system of measuring *in vivo* mediator release is convenient and offers the investigator the ability to carry out a variety of pharmacologic and clinical trials. The system does, however, have a number of disadvantages. First, the varying dilution created by nasal washes means that the absolute amount of mediator released cannot be quantitatively determined. Thus, the technique gives, at best, semiquantitative data. Another disadvantage is the need for the use of an α-adrenergic agonist. Although we have shown that the use of oxymetazoline hydrochloride did not alter the relative concentration of mediators or change the dose of antigen necessary to initiate sneezing, the presence of any drug makes the system less than ideal. Despite these disadvantages, it is the most flexible system presently available, and it has recently been used by us to demonstrate that an antimast cell release drug (azatadine, a tricyclic antihistamine) significantly blocks both the sneezing response and the release of mediators (30). These data will be presented elsewhere.

In sum, we have shown for the first time that one symptom of allergic rhinitis, sneezing, induced by antigenic challenge, is associated *in vivo* with the release of histamine and other putative mast cell mediators. In so doing, we have developed a highly reproducible and flexible system for the further study of the allergic response and nasal pathophysiology.

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